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APPLICATION FOR LETTERS PATENT

for

MEANS AND METHODS FOR TREATMENT EVALUATION

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TITLE OF THE INVENTION

MEANS AND METHODS FOR TREATMENT EVALUATION

CROSS-REFERENCE TO RELATED APPLICATIONS

Under the provisions of 35 U.S.C. 119(e), priority is claimed from U.S. Provisional Patent Application Serial No. 60/325,722, filed September 28, 2001.

TECHNICAL FIELD

The invention relates to the field of medicine. The invention particularly relates to the fields of molecular biology and detection methods.

BACKGROUND

Recent advances in the knowledge of molecular processes in a cell and techniques to study these processes have resulted in improved methods of typing and treating diseases. Understanding of the underlying molecular diversity of tumors has, for instance, already led to a better understanding of the diversity of response to treatment of morphologically similar tumors. Improved typing influences the way tumor patients are being treated. A drawback of the current methods of treatment is, however, that it takes a relatively long time to determine whether a treatment given to a patient is actually effective. This impedes the optimization of dosages and/or schedules with which treatment is given. Moreover, it also slows down the possibility of adjusting the treatment regimen all together. For instance, adjustment of therapy is currently only possible when macroscopic analysis of tumor cells in the body indicates that the therapy given is not effective. Macroscopic changes typically need several weeks to manifest themselves and equipment to measure such changes is often not readily available.

DISCLOSURE OF THE INVENTION

The present invention provides a method for determining whether a treatment is effective in changing a status of a certain set of target cells in an individual comprising obtaining a sample from the individual after initiation of the treatment and determining whether the sample comprises an expression product of at least one marker gene. In one embodiment of the

invention the set of target cells comprises a tumor cell. By changing a status of a set of target cells is meant herein that at least one property of the set of target cells is altered. For instance, the *amount* of the target cells may be changed. The amount may either be increased or decreased. Alternatively, the *activity* of the target cells may be altered. The activity may be a replication activity. As another example, the activity may be an activity involved with angiogenesis. Alternatively, the activity may be an apoptotic activity.

It was found that tumor cells and/or surrounding tissue respond, on a molecular level, very quickly to an effective treatment. This response can be detected by measuring an expression product of a marker gene. Marker gene expression products are indicative for a response to treatment. Marker genes are typically genes that are expressed by said set of target cells, for instance tumor cells, and/or surrounding tissue. However, marker genes can also be expressed in non-tumor target cells in other compartments of the body, for instance blood cells and/or cardiovascular cells.

Alternatively, marker gene expression can be initiated upon treatment given to the individual. Marker gene expression products are responsive to treatment given to a patient. A response can be an alteration in the relative amounts of expression product. However, it can also be an alteration in absolute presence or absence of expressed product such as RNA and/or protein.

According to the invention, a sample which is obtained from a patient may comprise at least one of the target cells. This is particularly suitable for detecting circulating tumor cells which have released themselves from a tumor and are circulating in the blood of a patient. Alternatively, the target cells may be non-tumor cells. In another embodiment of the invention, the sample does not comprise any target cells. However, the sample may comprise another, non-target cell. Expression, or change of expression, of at least one marker gene by the non-target cell is indicative for the status of a certain set of target cells. The non-target cell preferably comprises a peripheral blood mononuclear cell, as is described below. In yet another embodiment, the sample does not comprise any cell at all. For instance, an expression product of a marker gene, produced by a target cell or non-target cell elsewhere in an individual's body, may be present in the sample at detectable levels.

With a method of the invention it is possible to determine whether a treatment is effective in said individual. This can be done while a treatment is given or shortly after said treatment. Thus it is possible for instance to adjust treatment schedule, dosages and type on a patient per patient basis. It is preferred that said sample is obtained within a week of initiation of treatment. More preferably, said sample is obtained within two days of initiation of treatment. With a method of the invention it is possible to evaluate treatment effectiveness almost immediately after initiation of said treatment. A method of the invention thus offers a good opportunity for determining whether treatment adjustments are required.

A marker gene preferably comprises a gene involved in the generation, maintenance and/or breakdown of blood vessels (angiogenesis). Classes of genes involved in the process of angiogenesis encompass among others receptors, ligands and signaling molecules. Tumor cells are dependent on the growth of new blood vessels to maintain expansion of tumor mass. On the one hand, blood vessels are required to carry nutrients to the site of the tumor, whereas on the other hand waste material needs to be transported from the tumor. In the present invention it has been shown that expression products from genes involved in the generation, maintenance and breakdown of a blood vessel are among the first to respond to anti-tumor treatments. Such genes are therefore very suitable marker genes of the invention. In one embodiment said marker gene comprises a sequence as depicted in table 1 or 2. In another embodiment said marker gene comprises a sequence as depicted in figures 1-18, or a part or analogue thereof. In a preferred embodiment said marker gene comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof.

A change in the level of expression product of a marker gene is indicative for whether a treatment is effective or not. For instance, the level of expression product of a marker gene can be enhanced in a sample when a treatment is effective, alternatively expression product of a marker gene can

be reduced. Thus, preferably, expression product of a marker gene is quantified. The level of expression product in a sample can vary due to changes in the expression of a marker gene. However, it is also possible that the level changes due to a change in type of cells comprising said expression product in said sample, for instance due to treatment related cell death at the site of the body where the sample is obtained. Considering that the level of expression product of marker genes can vary from patient to patient, it is preferred that a method of the invention further comprises comparing the level of expression product of said marker gene with a reference. Preferably said reference comprises the same type of tumor cells prior to, or in the absence of, said treatment. Preferably, said tumor cells are derived from the same patient. The difference in the level of expression product of a marker gene in an effective and a non-effective treatment can be very large. In the extreme cases the level of expression product can range from detectable to not detectable. Marker genes displaying such zero to one relation in expression product levels are preferred in the present invention. A zero to one relation can be used to design relatively simple test systems. A zero to one relation is of course dependent on the detection system used to detect expression product of a marker gene. Very sensitive expression detection systems will typically detect expression product where a less sensitive systems detects no expression product. An expression product can be RNA or a part thereof, transcribed from said marker gene or a translated protein or a part thereof. A person skilled in the art is well capable of designing the most appropriate expression detection system to practice this preferred embodiment of the invention.

A part of an RNA or DNA molecule is defined herein as an RNA or DNA sequence, comprising at least 50 nucleotides. A part and/or an analogue of an expression product is defined herein as a part and/or analogue that can be detected using essentially the same kind of detection method as said expression product, although the sensibility of detection may differ. An analogue of an RNA or DNA molecule is defined herein as an RNA or DNA

sequence which is essentially the same as a particular RNA or DNA sequence. However, a nucleotide mutation, replacement, alteration, addition and/or deletion may have taken place naturally and/or performed artificially, without essentially altering the detection of said analogue as compared with the detection of said particular RNA or DNA sequence. A person skilled in the art is well able to determine whether a given RNA or DNA sequence is an analogue of a particular RNA or DNA sequence, using techniques known in the art.

In a preferred embodiment said tumor comprises Kaposi's Sarcoma. Kaposi's Sarcoma is a disease of proliferating blood vessels and therefore very much suited for identifying marker genes involved in angiogenesis. According to the invention, changes in angiogenesis factors are among the first marker events as a result of treatment. Kaposi's Sarcoma (KS) manifests itself clinically by reddish skin lesions. Kaposi's Sarcoma is a multicentric, malignant neoplastic vascular proliferation characterized by the development of bluish-red cutaneous nodules, usually on the lower extremities, most often on the toes or feet, and slowly increasing in size and number and spreading to more proximal areas. The tumors have endothelium-lined channels and vascular spaces admixed with variably sized aggregates of spindle-shaped cells, and often remain confined to the skin and subcutaneous tissue, but widespread visceral involvement may occur. Kaposi's Sarcoma occurs spontaneously in Jewish and Italian males in Europe and the United States. An aggressive variant in young children is endemic in some areas of Africa. A third form occurs in about 0.04% of kidney transplant patients. There is also a high incidence in AIDS patients. (From Dorland, 27th ed & Holland et al., Cancer Medicine, 3d ed, pp2105-7)

Kaposi's Sarcoma is aggressive in HIV infected individuals. The angiogenic mechanism causing the lesions results from the interplay of viral and cellular gene expression and is poorly understood in terms as to which genes are involved and what controls their expression. The angiogenic

proliferation in KS involves mechanisms likely to be universal in angiogenesis. The central role of angiogenesis in Kaposi's Sarcoma is clearly illustrated by the French name for this tumor: angiosarcomatose kaposi. Because of said central role of angiogenesis in Kaposi's Sarcoma, determination of marker genes involved in angiogenesis is very suitable to determine whether a treatment of Kaposi's Sarcoma is effective.

In the present invention, gene expression patterns of Kaposi's Sarcoma were examined with a method called serial analysis of gene expression (SAGE) (Velculescu et al. (1995) Science 270; 484-487). This method allows the quantitative and simultaneous analysis of a large number of transcripts. SAGE is based on two principles. First, a short nucleotide sequence TAG (14 base pairs) contains sufficient information to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. Second, concatenation of short sequence TAG's allows the efficient analysis of transcript in a serial manner by sequencing of multiple TAG's within a single clone.

Briefly, in this method a biotinylated oligo (dT) primer is used to synthesize cDNA from mRNA, and after digestion with a restriction enzyme, the most 3' terminus (near the poly-A tail) is isolated. These 3' fragments of cDNA are ligated to linkers and cleaved with a type II restriction enzyme to release short sequence (14 bp) of the original cDNA (TAG's). The TAG's are ligated to diTAG's and PCR amplified. These di-TAG's are then ligated to form long concatamers, which are cloned and sequenced. In this way, one sequence reaction yields information about the distribution of many different mRNA's. Finally, the calculation of the abundance of different TAG's and the matching of the TAG's in Genbank are done using the necessary computer software.

In another aspect the invention provides the use of a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, or a part or analogue thereof, in an expression product detection method. Preferably

said nucleic acid comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. Expression of a marker gene in an individual can be detected by determining whether said nucleic acid or part or analogue is able to hybridize with nucleic acid, preferably RNA, in a sample of said individual. If hybridisation takes place, it is indicative of expression of a marker gene in said individual. Of course, as is known by a person skilled in the art, a coding strand of DNA/RNA is capable of hybridizing with the complementary strand of a corresponding doublestranded nucleic acid sequence. Hence, a complementary strand of a certain coding strand is particularly suitable for detection of expression of said coding strand. For instance, a complementary strand of a coding strand as depicted in figure 1-18 and/or table 1 or 2 is suitable for detection of expression of a gene comprising said coding strand.

In yet another aspect, the invention provides the use of a proteinaceous molecule capable of specifically binding a protein encoded by a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, or a part or analogue thereof, in a detection method. Preferably, said proteinaceous molecule is capable of specifically binding a protein encoded by a nucleic acid comprising a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. In one embodiment of the invention, said uses are directed toward determining the presence of a site of angiogenesis in an individual. In another embodiment of the invention, said uses are directed toward determining the presence of a tumor cell in an individual. The presence of a tumor cell in an individual can be determined because said tumor cell typically expresses marker genes that can be detected by an expression product detection method. For instance, an antibody, or analogue thereof, specifically directed against an expression product of said marker gene can be generated. Said antibody or analogue is suitable for determination of an expression product of said marker gene in a sample. To determine the presence of a tumor cell in an individual, a sample

from said individual can be incubated with said antibody. If said sample contains an expression product of said marker gene, said antibody will bind. Binding can be demonstrated by techniques known in the art, like for instance ELISA. If binding of said antibody is demonstrated, one can conclude that said sample contains an expression product of said marker molecule. The presence of an expression product of said marker molecule can indicate the presence of a tumor cell in an individual, since said marker molecule is expressed by tumor cells. There are of course many more alternative techniques to detect an expression product with use of a proteinaceous binding molecule, which are well known in the art and need no further discussion here. Thus, proteins expressed by a tumor cell can be detected by a proteinaceous molecule capable of specifically binding a protein encoded by a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, like for instance a TIE 1 sequence, a Salioadhesin and/or Siglec 1 sequence, or a part or analogue thereof. Likewise, the presence of a site of angiogenesis in an individual can be determined by detecting an expression product of a marker gene.

In another embodiment, a use of a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, or a use of a proteinaceous molecule capable of specifically binding a protein encoded by a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, or a part or analogue thereof, are directed toward determining whether a treatment is effective in changing the status of a certain set of target cells in an individual. In a preferred embodiment said nucleic acid comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. In another preferred embodiment said proteinaceous molecule is capable of specifically binding a protein encoded by a nucleic acid comprising a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. More preferably, said uses are directed toward determining whether a treatment is

effective in counteracting a tumor in an individual. In one embodiment of the invention, said tumor comprises Kaposi's Sarcoma.

In one aspect the invention provides the use of a nucleic acid comprising a sequence as depicted in table 1 or 2 and/or figure 1-18 as an indicator for angiogenesis. In a preferred embodiment the invention provides the use of a nucleic acid comprising a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof as an indicator for angiogenesis. For instance, a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2 can be used as detection marker for the process of angiogenesis in the course of regenerative treatment. Changes in the expression level of the detection marker indicate active growth of blood vessels (i.e. angiogenesis) as was meant to induce with the regenerative treatment course. In a preferred embodiment such application is in the field of heart and coronary disease aimed at generation of new blood supply to affected organs by means of new blood vessels. Likewise, the treatment of tumors with anti-angiogenesis drugs can be monitored by changes in expression levels of detection marker genes as depicted in figure 1-18 and/or table 1 or 2, such as a TIE 1 sequence, a Salioadhesin and/or Siglec 1 sequence.

In another aspect the invention provides the use of a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2 as detection marker for tumor cells. In yet another aspect the invention provides the use of a proteinaceous molecule encoded by a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2 or a proteinaceous molecule capable of binding a protein encoded by a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2 as detection marker for tumor cells.

With a method of the invention it is possible to monitor a specific status of an individual. The presence of a disease – or danger of developing one – can be determined by determining whether or not a sample of an individual comprises an expression product of a marker gene. This means that also the absence of a marker gene in a sample can be indicative for the presence of a disease, or for danger of developing a disease. This is possible for any disease, as long as the disease involves an altered expression pattern of at least one marker gene. Preferably the presence of a marker gene in a sample is determined.

Additionally, a healing process can be followed as well. For instance, recovery of damaged tissue can involve an increasing amount of expression product of a marker gene over time. It is however also possible that recovery of damaged tissue involves a decreasing amount of expression product of a marker gene over time. Samples taken at different time intervals provide information about the amount of expression product which is generated at different time points. An altered amount of a specific expression product found in samples during a period of time is indicative of the amount of tissue cells generated. Likewise, a decreasing amount of an expression product found in samples in a specific time-period can indicate a certain – either beneficial or harmful – process. For instance, said process may involve the development or the treatment of disease. An important application is a treatment of heart and coronary disease. A method of the invention is very suitable for monitoring the generation of new cardiac tissue.

Thus, one aspect of the invention provides a method of diagnosis, in particular a method for determining whether an individual comprises a tumor cell and/or a site of angiogenesis, comprising obtaining a sample from an individual, and determining whether said sample comprises an expression product of at least one marker gene. Preferably, said marker gene comprises a sequence as depicted in table 1 or 2 and/or figure 1-18, or a part or analogue

thereof. More preferably said marker gene comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. With a method of the invention it is possible to detect tumor cells that have released themselves from the tumor and are elsewhere in the body. In a preferred embodiment such detection is performed in the blood of a person detecting circulating tumor cells. These circulating tumor cells can be used for primary identification of presence of a tumor somewhere in the body and also for identification of the risk of metastasis of the tumor to other places in the body next to the primary location of the body. Likewise, a method of the invention is suitable for determining a site of angiogenesis in an individual. Angiogenesis is an indicator for different aspects. For instance, an increased level of angiogenesis indicates the presence of tumor cells, or the healing of damaged tissue, like for instance recovery of heart and coronary disease.

Since an angiogenic process is now easily monitored by a method of the invention, it is likewise easy to determine whether a certain treatment is effective in altering an angiogenic process. For instance, if a certain treatment is effective in counteracting an angiogenic process, the amount of an expression product of a marker gene involved in angiogenesis decreases as well over time. In the art, many drugs are known for anti-angiogenic treatment. Thus, one embodiment of the invention provides a method for determining whether a treatment is effective in altering an angiogenic process in an individual comprising obtaining a sample from said individual after initiation of said treatment, and determining whether said sample comprises an expression product of at least one marker gene. Preferably, said marker gene comprises a sequence as depicted in table 1, 2, and/or figures 1-18, or a part or analogue thereof. More preferably, said marker gene comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. In one embodiment, said treatment comprises counteracting angiogenesis in said individual. In yet another

embodiment said treatment involves the use of at least one of the following drugs: 2ME2, Angiostatin, Angiozyme, Anti-VEGF RhuMAb, Apra (CT-2584), Avicine, Benefin, BMS275291, Carboxyamidotriazole, CC4047, CC5013, CC7085, CDC801, CGP-41251 (PKC 412), CM101, Combretastatin A-4 Prodrug, EMD 121974, Endostatin, Flavopiridol, Genistein (GCP), Green Tea Extract, IM-862, ImmTher, Interferon alpha, Interleukin-12, Iressa (ZD1839), Marimastat, Metastat (Col-3), Neovastat, Octreotide, Paclitaxel, Penicillamine, Photofrin, Photopoint, PI-88, Prinomastat (AG-3340), PTK787 (ZK22584), RO317453, Solimastat, Squalamine, SU 101, SU 5416, SU-6668, Suradista (FCE 26644), Suramin (Metaret), Tetrathiomolybdate, Thalidomide, TNP-470, and/or Vitaxin. However, the artisan can think of more drugs that can be used during said treatment.

In a preferred embodiment, a sample of a method of the invention is a blood sample. Although the location of for instance an angiogenic process can be a tumor or a part of the skin, a blood sample is preferred, among other things because it is much easier to obtain. A blood sample is also often easier to investigate, requiring less expensive and/or specific equipment. Quite surprisingly, we have found that the expression of certain marker genes by hemopoietic cells, like peripheral blood mononuclear cells (PBMC), can be indicative for a process occurring somewhere else in an individual's body. For instance, the presence, or alteration in amount, of an expression product of a marker molecule in PBMC can indicate the presence of a tumor somewhere in the body. In example 10 it is for instance shown that a TIE 1 sequence (tag 15, table 3) or a Salioadhesin or Siglec 1 sequence (tag 32, table 4) are both upregulated in skin tumor and in PBMC cells in a Kaposi's Sarcoma patient. Additionaly, example 8 shows that the absence of expression product of a Keratin 14 sequence (tag 7, table 3) in a blood sample of said patient, whereas Keratin 14 is overexpressed in tumor cells, indicates that said sample was not contaminated with tumor cells. Likewise, the expression of certain marker

genes by PBMC can provide another diagnostic indication. The presence or absence of an expression product of a marker gene in PBMC provides adequate information about different aspects and/or processes of an individual's body. Preferably, the amount of expression product in a non-hemopoieteic cell is compared with a reference value. This way an indication is obtained about an increment or decrement of expression in said hemopoietic cell.

In one aspect the invention therefore provides a method for determining whether an individual comprises a non-hemopoietic tumor cell and/or a site of angiogenesis, said method comprising determining whether a hemopoietic cell from said patient comprises an altered amount of an expression product of a marker gene as compared with a reference value. Preferably, said marker gene comprises a gene involved in angiogenesis. More preferably, said gene comprises a sequence as depicted in table 1 or 2, and/or figures 1-18, or a part or analogue thereof. Most preferably, said gene comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. In one embodiment of the invention said hemopoietic cell comprises a peripheral blood mononuclear cell.

In one aspect the invention provides a method of the invention, wherein said expression product is expressed by a PBMC. Preferably, said expression product comprises a TIE 1 sequence, a Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 8 or 17, or a part or analogue thereof. As these sequences are involved in angiogenesis, the invention also provides a use of a PBMC expressed Keratin 14 sequence, TIE 1 sequence, Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 2, 8 or 17, or a part or analogue thereof, as an indicator for angiogenesis. Likewise, these sequences are involved in the presence of tumor cells. Therefore, a use of a PBMC expressed Keratin 14 sequence, TIE 1 sequence, Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 2, 8, or 17, or a part or analogue

thereof, for determining the presence of a tumor cell in an individual, is also herewith provided. Additionally, the invention also provides an isolated Keratin 14 sequence, TIE 1 sequence, Salioadhesin or Siglec 1 sequence, a sequence as depicted in figure 2, 8, or 17, or a part or analogue thereof, for use in a diagnostic method. A diagnostic method can be carried out using a diagnostic kit. Therefore, a diagnostic kit comprising a nucleic acid comprising a sequence as depicted in figure 1-18 and/or table 1 or 2, or a part or analogue thereof, and/or a proteinaceous molecule capable of specifically binding a protein encoded by said nucleic acid or said part or analogue, is also herewith provided. Preferably, said kit comprises a suitable means of detection. In one embodiment a diagnostic kit of the invention is provided comprising a Keratin 14 sequence, and/or a TIE 1 sequence, and/or a Salioadhesin or Siglec 1 sequence, and/or a sequence as depicted in figure 2, 8 or 17, or a part or analogue thereof.

A diagnostic kit of the invention is particularly useful for carrying out a method of the invention. In yet another embodiment the invention therefore provides a use of a diagnostic kit of the invention for determining whether a treatment is effective in changing the status of a certain set of target cells in an individual and/or altering an angiogenic process in an individual.

Additionally, the invention provides a use of a diagnostic kit of the invention for determining whether an individual comprises a tumor cell and/or a site of angiogenesis.

With a marker gene of the invention it is possible to screen for drugs directed against a disease for which said marker gene is indicative. There are many methods available in the art for screening for a specific drug activity. For instance, cells can be incubated with different potential drug compounds, and an expression pattern of a marker gene in said cells before and after exposure to each potential drug compound can be compared. A specific difference in an expression pattern after exposure to a particular potential drug compound shows that said compound is a suitable candidate for the

development of a medicament. The invention therefore provides in one embodiment a use of an expression product of a gene comprising a sequence as depicted in figure 1-18, table 1 or table 2 as a drugtarget. Preferably, said sequence is a Salioadhesin or Siglec 1, TIE 1, and/or Keratin 14 sequence. A compound capable of altering the activity of Salioadhesin or Siglec 1, TIE 1, Keratin 14, and/or the expression of Salioadhesin or Siglec 1, TIE 1, and/or Keratin 14 in a cell is also herewith provided. Said compound is particularly suitable for the preparation of a medicament.

The present invention is further explained in more detail by the following examples, which do not limit the invention in any way.

Examples

Example 1

In this example a selection of samples for analysis of expression profiles is made.

A 31-year old man was demonstrated to be HIV-1 seropositive in February 1997. The initial CD4 cell count was 25 x 10^6/ l. The patient presented within two months a mucocutanous Herpes simplex infection and an extrapulmonary Cryptococcosis for which specific medication was given. The HIV-1 RNA load at presentation was 15,000 copies/ml and increased to 33,000 copies/ml in three months. Then antiretroviral therapy was started with zidovudine, lamivudine and indinavir. Immediately after start therapy the HIV-1 RNA load dropped below detection limit. In November 1997 the patient presented with gradual appearance of an increasing number of violaceous skin lesions that clinically resembled Kaposi's Sarcoma. The diagnosis was confirmed by histological examination of one of the lesions. At start of the chemotherapy (bleomycin, vincristine and adriamycine intravenously) KS had progressed to about 150 cutaneous lesions. The interval between the courses of chemotherapy was three weeks and stopped after the fifth course. Several lesions had disappeared by three weeks of therapy and complete remission was gradually reached after one year.

During chemotherapy several biopsies were taken. The first biopsy was obtained 24 hours after start chemotherapy (named KS1) and the second biopsy after 48 hours (named KS2). All biopsies were flash-frozen in liquid nitrogen immediately after surgical removal and stored at -80°C. Diagnosis of Kaposi's Sarcoma was confirmed histopathologically.

Control SAGE libraries KS3 and KS4 were made from frozen material taken at autopsy from two AIDS patients with Kaposi's Sarcoma, both of which died in 1986 without having had any form of chemotherapy or retroviral treatment.

Example 2

The expression profiles of the biopsy samples were determined using the SAGE technology. All biopsies were cut with a microtome in 15-20 μm sections and transferred to a tube containing TRIzol. RNA isolation with TRIzol was performed according to the manufacturer's instructions. Poly (A) RNA was obtained using the Micro-FastTrack™ 2.0 mRNA Isolation Kit. cDNA preparation and the subsequent steps were performed as described by Velculescu. Primary analysis of the sequence results was performed using software especially designed for SAGE by the Bioinformatics Laboratory of the Academic Medical Centre, Amsterdam (van Kampen *et al.* USAGE: a web-based approach towards the analysis of SAGE data. Bioinformatics, *in press*). The libraries were also analysed using the Human Transcriptome Map (HTM), a program developed in the AMC, which maps TAG's onto human chromosomes (Caron *et al.* The Human Transcriptome Map reveals a clustering of highly expressed genes in chromosomal domains. Submitted for publication).

We sequenced ~ 47,000 TAG's from the four biopsies, 47,298 TAG's from KS 1 library, 46,671 from the KS 2 library, 49,335 TAG's from the KS3 library, and 48,814 TAG's from the KS4 library. TAG lists (i.e. individual TAG's plus the number of appearance) were compared with each other in USAGE, TAG sequences with the highest counts were identified with the amct2g database available in USAGE (which is an improved TAG identification compared with the SAGEmap database from CGAP (available from GenBank). Secondly, TAG lists were mapped to chromosome locations with the HTM program, and at the

same time compared with specific TAG lists (e.g. vascular endothelium, publicly available), and with a compilation of all TAG lists in the SAGEmap database (designated "All" in HTM) TAG's belonging to genes specifically up regulated in KS3 and KS 4 identified (Table 1). Nucleotide 15 was determined from the original diTAG list in USAGE. The TAG sequence of 15 nt. was checked with GenBank (BLAST) to confirm its identification. A few TAG's were eliminated because of ambiguity in the 15^{th} nucleotide, or because of misidentification.

Example 3

Result of the analysis showing the identifiable TAG's derived from known genes with increased expression in Kaposi's Sarcoma SAGE libraries KS3 and KS4 compared to libraries KS1 and KS2. The TAG numbers are first normalized to a level of 100.000 total TAG's sequenced per library to allow comparison between the libraries and comparison with other libraries in the public domain.

The sequence catg precedes each TAG sequence given in column 2 of table 1.

Table 1. Overview of identifiable TAG's over-expressed in SAGE libraries KS3 and KS4.

No.	TAG sequence (5' -> 3')	Unigene no.	ID	overexpression factor[1]
1.	ccccagtcggc	Hs171596	EphA2	3
2.	cttgacatacc	Hs171695	Dual specificity phosphatase	3
3.	catcacggatc	Hs82112	IL1 receptor, type 1[2]	10-30
4.	ggccaaaggcc	Hs78436	EphB1	>2
5.	ttgcatatcag	Hs82237	AT group D protein	10 - 15
6.	ccctgttcagc	Hs78824	Tie 1[2]	2-5
7.	gatcaatcagt	Hs16530	Small ind. cytokine A18	10-20
8.	gagggtgccaa	Hs898	Complement comp. 1Qβ	5-10
9.	taaacctgctg	Hs99923	galectin 7	3-10
10.	gtggccagagg	Hs1420	FGFR3	2-5
11.	tctggcccagc	Hs183	DARC (Duffy blood group)	8-10
12.	caggtcgctac	Hs75066	Translin	2-6
13.	gagcagcgccc	Hs112408	Psoriasin (S100 A7)	> 20 (specific)
14.	acttattatgc	Hs76152	Decorin	2-10
15.	caggcctggcc	Hs74649	Cytochrome C oxydase subunit VIc	2-4
16.	gtgcggaggac	Hs181062	Serum amyloid A1	5-14
17.	acagcggcaat	Hs74316	Desmoplakin	5-10
18.	gatgtgcacga	Hs117729	Keratin 14	10-14
19.	caggtttcata	Hs24395	Small ind. cytokine, B14 (BRAK)	5-10
20	aactctgaccc	Hs93675	Decidual protein induced by progesterone[2]	3-10

1. TAG numbers of appearance were normalized to library sizes of 100 000 TAG's.

2. Identified as Pan Endothelial Markers by St. Croix *et al.*, Genes expressed in human tumor endothelium. *Science* 289:1197-1202, 2000.

Example 4

Result of the analysis showing the non-identifiable TAG's derived from EST's of genes with unknown function with increased expression in Kaposi's Sarcoma SAGE libraries KS3 and KS4 compared to libraries KS1 and KS2. The TAG numbers are first normalized to a level of 100.000 total TAG's sequenced per library to allow comparison between the libraries and comparison with other libraries in the public domain.

The sequence catg precedes each TAG sequence given in column 2 of table 2.

Table 2. Overview of identifiable TAG's over-expressed in SAGE libraries KS3 and KS4.

No.	TAG sequence (5' -> 3')	Unigene no.	ID	overexpression factor[1]
1.	aaatcaataca	Hs94953	EST	4-10
2.	tggtaactggc	Hs108741	EST	4-10
3.	tctgcactgag	Hs173789	EST	2-4
4.	caggctgctgg	Hs60440	EST	4-30
5.	atgacagatgg	Hs13775	EST	5-10
6.	gcacaacaaga	Hs236510	EST	3-10
7.	ccacaggagaa	Hs23579	EST	4-10
8.	ctgtgcggaac	Hs46987	EST	2-10
9.	gatggctgcct	Hs18104	EST	4-20
10.	ctccattgcca	Hs31869	EST	2-10
11	acctccactgg	Hs112457	EST	Unique[2]

1. TAG numbers of appearance were normalized to library sizes of 100.000 TAG's
2. This TAG does not appear in any other SAGE library than our own libraries and seems to be a unique new indicator gene for angiogenesis.

Example 5

Kaposi's sarcoma skin tissue was obtained from the same two AIDS patients mentioned in example 1 from whom SAGE libraries KS3 and KS4 were made. Both patients were homosexual men and were infected at the beginning of the HIV-1 epidemic in Europe. Patient 1 born in Indonesia was demonstrated to be HIV-1 positive in 1982. In February 1985 a histological examination confirmed the diagnosis of Kaposi's sarcoma. He died 13 month later and postmortem examination revealed morphological variants of visceral KS. Patient 2 presented in February 1984 at the Academic Medical Centre with progressive KS skin lesions. During follow-up the KS progressed to the intestines, oropharynx, lung, tongue, sinus piriformis and lymph nodes. In March 1986 the patient died and autopsy took place. The biopsies of said two patients were named KS3 and KS4.

Normal adult breast skin tissue was obtained as discarded tissue from reduction mammoplasties (obtained from the department of plastic Surgery of our hospital). RNA isolated of three breast reductions was used to construct the normal skin expression profile library.

The expression profiles of the biopsy samples were determined using the SAGE technology as described in example 2.
We sequenced ~ 47,000 TAG's from the four biopsies, 49,335 TAG's from the KS3 library, and 48,814 TAG's from the KS4 library. TAG lists (i.e. individual TAG's plus the number of appearance) were compared with each other in USAGE, TAG sequences with the highest counts were identified with the amct2g database available in USAGE (which is an improved TAG identification compared with the SAGEmap database from CGAP (available from GenBank). Secondly, TAG lists were mapped to chromosome locations with the HTM program, and at the same time compared with specific TAG

lists (e.g. vascular endothelium, publicly available), and with a compilation of all TAG lists in the SAGEmap database (designated "All" in HTM) TAG's belonging to genes specifically up regulated in KS3 and KS 4 identified (Table 1). Nucleotide 15 was determined from the original diTAG list in USAGE. The TAG sequence of 15 nt. was checked with GenBank (BLAST) to confirm its identification. A few TAG's were eliminated because of ambiguity in the 15^{th} nucleotide, or because of misidentification.

Example 6

Result of the analysis showing the identifiable TAG's derived from known genes with increased expression in Kaposi's Sarcoma SAGE libraries KS3 and KS4 compared to the public libraries of National Center for Biotechnology Information. The TAG numbers are first normalized to a level of 100.000 total TAG's sequenced per library to allow comparison between the libraries and comparison with other libraries in the public domain.

The sequence catg precedes each TAG sequence given in column 2 of table 3.

Table 3. Overview of identifiable tag's overexpressed in SAGE libraries KS3 and KS4

Tag number	Tag sequence	Unigene no.	ID	overexpressed
TAG007	gatgtgcacga	Hs117729	Keratin 14	10-14
TAG010	ccccagtcggc	Hs171596	Eph A2 (angiogenesis)	3
TAG011	cttgacatacc	Hs171695	Dual specificity phosphatase	3
TAG012	catcacggatc	Hs82112	IL1 receptor, type 1*	10-30
TAG013	ggccaaaggcc	Hs78436	Sorting nexin 17	>2
TAG014	ttgcatatcag	Hs82237	AT group D protein	10 – 15
TAG015	ccctgttcagc	Hs78824	Tie 1*(angiogenesis)	2-5
TAG016	gatcaatcagt	Hs16530	Small ind. cytokine A18	10-20
TAG017	gagggtgccaa	Hs898	Complement comp. 1Qß	5-10
TAG018	taaacctgctg	Hs99923	galectin 7(specific)	3-10
TAG019	gtggccagagg	Hs1420	FGFR3 (activated in carcinomas, angiogenesis)	2-5
TAG020	tctggcccagc	Hs183	DARC (Duffy blood group)	8-10
TAG021	caggtcgctac	Hs75066	Translin(involved in translocations)	2-6
TAG022	gagcagcgccc	Hs112408	Psoriasin (S100 A7)	> 20 (specific)
TAG033	acttattatgc	Hs76152	Decorin (connective tissue)	2-10
TAG034	caggcctggcc	Hs288761	Hypothetical protein FLJ21749	2-4
TAG035	gtgcggaggac	Hs181062	Serum amyloid A1	5-14
TAG036	acagcggcaat	Hs74316	Desmoplakin	5-10
TAG037	caggtttcata	Hs24395	Small ind. cytokine, B14 (BRAK)	5-10
TAG038	aactctgaccc	Hs93675	Decidual protein induced by progesterone*	3-10

*Identified as Pan Endothelial Markers by St. Croix *et al.*, Genes expressed in human tumor endothelium. *Science* **289**:1197-1202, 2000.

Example 7

Result of the analysis showing the non-identifiable TAG's derived from EST's of genes with unknown function with increased expression in Kaposi's Sarcoma SAGE libraries KS3 and KS4 compared to libraries KS1 and KS2. The TAG numbers are first normalized to a level of 100.000 total TAG's sequenced per library to allow comparison between the libraries and comparison with other libraries in the public domain.

The sequence catg precedes each TAG sequence given in column 2 of table 4.

Table 4.
Overview of identifiable tag's over-expressed in SAGE libraries KS3 and KS4

Tag number.	Tag sequence (5'>3')	Unigene no.	ID	overexpressed
TAG023	aaatcaataca	Hs94953	EST	4-10
TAG024	tggtaactggc	Hs108741	EST	4-10
TAG025	tctgcactgag	Hs173789	EST	2-4
TAG026	caggctgctgg	Hs60440	EST	4-30
TAG027	atgacagatgg	Hs13775	EST	5-10
TAG028	gcacaacaaga	Hs236510	EST	3-10
TAG029	ccacaggagaa	Hs23579	EST	4-10
TAG030	ctgtgcggaac	Hs46987	EST	2-10
TAG031	gatggctgcct	Hs18104	EST	4-20
TAG032	ctccattgcca	Hs31869	EST	2-10
TAG004	acctccactgg	Hs112457	EST	Unique*

*This tag does not appear in any other SAGE library than our own libraries and seems to be unique new indicator gene for angiogenesis,.

The overexpressed TAG's listed in tables 3 and 4 are the same as in tables 1 and 2, respectively. This shows that the expression pattern after treatment is

comparable with the expression pattern of healthy individuals with normal expression patterns.

Example 8

Using an RT-PCR based method we were able to determine that TAG 11 (table 2) / TAG 004 (table 4) indeed represents a differently expressed gene. RNA was isolated from a KS lesion and the first strand cDNA synthesis was primed with an oligo(dT) primer with a 5' M13 tail (5'CTA GTT GTA AAA CGA CGG CCA G-$(T)_{24}$ 3'). Ten microliter total RNA was used, plus primer and 5 µl RT-mix (50 mM Tris, pH 8.3, 75mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 80 mM dNTPs and 20 units RNAsin were added, followed by an incubation for 3 minutes at 65°C and chilled on ice. The RT reaction starts by adding 5 units AMV RT followed by an incubation of 45 minutes at 42°C. For the PCR we used a 19-base TAG-specific primer (which consisted of 11 nt identified in the sage with a 5' NLAIII restriction site and 5 inosine nucleotides to increase the annealing temperature of the primers) and the –21M13 primer. The RT-mix was added to 80 µl PCR mixture containing the 100 ng of each primers (-21m13 PRIMER: 5' GTA AAA CGA CGG CCA GT 3' and 5' III IIC ATG ACC TCC ACT GG 3'), 50mM Tris (pH8.3), 20 mM KCl, 0.1 mg BSA per ml, dNTPs (0.1 mM each), 2,4 mM $MgCl_2$, and 2 units Taq polymerase. After incubation of 5 minutes at 94°C, the reaction was subjected to 35 cycles of amplification in a thermocycler (9700 Perkin-Elmer). A cycle included denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C and extension for 2 minutes at 72°C. The last cycle was followed by 72°C incubation for 10 minutes.

The amplified fragment was cloned in to an AT plasmid (InvitroGen) and subsequently the insert was sequenced using the dye terminator sequencing kit from Applied Biosystems Inc. The fragment appeared to have a length of

102 base pairs and the sequence analysis of the fragment revealed the sequence as depicted in figure 1. This sequence was identical to an EST sequence identified from human foetal heart (GenBank acc. # AI217565 and others), which in turn matched a predicted exon on chromosome 19. A relation with angiogenesis has not been described previously and is new.

Example 9

Confirmation of the identity of tag sequences.

A sequence consisting of 15 nucleotides should be enough to identify a particular specific mRNA or gene. To confirm the identity of the tags we developed a RT-PCR using an oligo24dT primer for the RT-reaction. A 5'primer containing the tag sequence itself is used for second strand synthesis. The oligo24dT primer is extended at the 5'site extended with a –21M13 sequence. In the PCR following the RT-reaction –21M13 primer is used for the amplification together with the 5'primer containing the tag sequence. The 5'primer containing the tag sequence is extended with 5 Inosines at its 5'site to enlarge the binding capacity of this primer. The sequence of the amplified fragment can be determined to confirm that this is the gene as identified by the tag sequence. The RT-PCR reactions to confirm the tags were performed on the same tissue samples that were used to prepare the expression profiles of tag sequences.

Procedure:

Common buffers used throughout the experiments:

10x RT buffer:

- 500 mM TRIS, pH 8.3
- 750 mM KCL
- 30 mM $MgCl_2$

- 100 mM DTT

10x PCR buffer:
- 200 mM TRIS pH 8.3
- 500 mM KCL
- 1 mg/ml BSA

1 ml TRIzol reagent (Invitrogen Life Technologies, cat. no. 15596) is added to 10-100 mg tissue or approximately 10^7 cells immediately (tissue is sliced 14µm thick by microtome).
The Total RNA isolation of the samples is performed according to the manufacturers protocol as follows:

- Add 0.2 ml of Chloroform (Merck) and shake the tube vigorously by hand for 15 seconds.
- Incubate for 5 minutes at RT.
- Centrifuge the sample at no more than 12,000 x g for 15 minutes at 4°C.
- Transfer 600µl of the colourless upper aqueous layer to a new tube. The lower organic layer should be red.
- Ad 0.5 ml isopropyl alcohol (Merck) and mix.
- Incubate at room temperature for 10 minutes.
- Centrifuge at no more than 12,000 x g for 15 minutes at 4°C.
- Discard the supernatant and wash the RNA pellet with 1 ml 80% ethanol by vortexing and centrifuge at no more than 7,500 x g at 4°C for 5 minutes and discard the supernatant.
- Place the tube at 56°C for 3 minutes to dry the pellet and proceed with the Poly A^+ RNA isolation as described in the next section.

The Poly A^+ mRNA isolation was performed according to the manual of Micro Fasttrack © 2.0 Poly A^+ mRNA 2.0 isolation kit as provided by the manufacturer (Invitrogen Corporation, Carlsbad USA; cat no K1520).

Subsequently the isolated poly A+ RNA was used as input for analysis in a RT-PCR reaction. The RT-PCR reactions started with the following mixture of ingredients:

21M13POLYT primer (100 ng/µl)	1.25 µl
10 x RT buffer	2.0 µl
100 mM dNTP (Pharmacia)	0.8 µl
20 U RNAsin (Roche)	0.3 µl
dH_2O (Baker)	0.65 µl

- Add 10 µl of Poly A^+ mRNA dilution to 5 µl of RT-mix.
- To anneal the primer to the template incubate the reaction mixture at 65°C for 5 minutes followed by cooling down to room temperature.
- Add 5µl 1U/µl AMV-RT to the reaction mixture and perform the Reverse Transcription by incubating at 42°C for 45 minutes.
- After the Reverse Transcription immediately incubate the mixture at 95°C for 5 minutes to stop the reaction. Then let the reaction cool down to room temperature.
- Add 80 µl of PCR-mix to each reaction mixture (total volume is 100 µl). Prepare the PCR-mix per reaction as follows:

- 5' primer (100 ng/µl), see table 5	1.0 µl
- 21M13 (100 ng/µl)	1.0 µl
- 10x PCR buffer	8.0 µl
- 100 mM $MgCl_2$	2.1 µl
- Amplitaq 5U/µl (Perkin Elmer)	0.4 µl
- dH_2O (Baker)	67.5µl

- PCR amplification was performed in a 9700 DNA thermal cycler (Perkin Elmer) according to the following program.

- 5 minutes 95 °C
- 1 minute 95 °C; 1 minute 55 °C; 2 minutes 72 °C, for 35 cycles
- 10 minutes 72 °C

The TA-cloning of the RT-PCR products was performed according to the manual of TOPO TA Cloning® kit as provided by the manufacturer (Invitrogen Corporation, Carlsbad USA; cat no K4600) using the pCR® II-TOPO® Dual promoter vector and the TOP10 One Shot® Cells.
Screening of the clones was performed using PCR with SP6 and T7 primers. As follows:

- Resuspend the colony in 50µl dH_2O (Baker). Add 1 µl of this bacteria suspension to 10 µl of PCR mixture.
- Prepare the PCR-mixture per reaction as follows:

SP6 (100 ng/µl)	0.10 µl
T7 (100 ng/µl)	0.10 µl
10x PCR buffer	1.00 µl
100 mM $MgCl_2$	0.20 µl
100 mM dNTP's (Pharmacia)	0.08 µl
Amplitaq 5U/µl (Perkin Elmer)	0.04 µl
dH_2O (Baker)	7.48 µl

- PCR amplification was performed in a 9700 DNA thermal cycler (Perkin Elmer) according to the following program.

- 5 minutes 95 °C
- 30 seconds 95 °C; 30 seconds 55 °C; 1 minutes 72 °C, for 25 cycles
- 10 minutes 72 °C

- Run 5 µl of the Colony-PCR product on a 1.5% agarose 1xTBE gel stained with EthidiumBromide.

- Visualise the amplification products on a UV-illuminator to identify insert-containing clones.

Clones containing insert were sequenced from both directions using SP6 and T7 primers and the ABI Prism Big-Dye terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster City, Calif USA).

Table 5: Primers used for tag confirmation

Tag name	Sequence	ID
-21M13POLYT	CTA GTT GTA AAA CGA CGG CCA GTT TTT TTT TTT TTT TTT TTT TTT T	RT-primer
TAG007	III IIC ATG GAT GTG CAC G	keratin 14
TAG010	III IIC ATG CCC CAG TCG GC	ephrin A2 (angiogenesis)
TAG011	III IIC ATG CTT GAC ATA CC	dual specificity phosphatase
TAG012	III IIC ATG CAT CAC GGA TC	IL1 receptor, type 1
TAG013	III IIC ATG GGC CAA AGG CC	Sorting Nexin 17 (SNX17)
TAG014	III IIC ATG TTG CAT ATC AG	AT group D protein
TAG015	III IIC ATG CCC TGT TCA GC	Tie 1 (angiogenesis)
TAG016	III IIC ATG GAT CAA TCA GT	small ind. Cytokine A18
TAG017	III IIC ATG GAG GGT GCC AA	complement comp. 1Q beta
TAG018	III IIC ATG TAA ACC TGC TG	galectin 7 (11 nt tag)
TAG019	III IIC ATG GTG GCC AGA GG	FGFR3 (11 nt tag)
TAG020	III IIC ATG TCT GGC CCA GC	DARC
TAG021	III IIC ATG CAG GTC GCT AC	Translin
TAG022	III IIC ATG GAG CAG CGC CC	Psoriasin (S100 A7) (11 nt tag)
TAG033	III IIC ATG ACT TAT TAT GC	Decorin
TAG034	III IIC ATG CAG GCC TGG CC	Hypothetical protein FLJ21749
TAG035	III IIC ATG GTG CGG AGG AC	Serum amyloid
TAG036	III IIC ATG ACA GCG GCA AT	Desmoplakin
TAG037	III IIC ATG CAG GTT TCA TA	Small ind. Cytokine, B14 (BRAK)
TAG038	III IIC ATG AAC TCT GAC CC	Decidual protein induced by progesterone
TAG023	III IIC ATG AAA TCA ATA CA	EST Unigene no. Hs94953
TAG024	III IIC ATG TGG TAA CTG GC	EST Unigene no. Hs108741
TAG025	III IIC ATG TCT GCA CTG AG	EST Unigene no. Hs173789
TAG026	III IIC ATG CAG GCT GCT GG	EST Unigene no. Hs60440
TAG027	III IIC ATG ATG ACA GAT GG	EST Unigene no. Hs13775
TAG028	III IIC ATG GCA CAA CAA GA	EST Unigene no. Hs236510
TAG029	III IIC ATG CCA CAG GAG AA	EST Unigene no. Hs23579 (PIG)
TAG030	III IIC ATG CTG TGC GGA AC	EST Unigene no. Hs46987
TAG031	III IIC ATG GAT GGC TGC CT	EST Unigene no. Hs18104
TAG032	III IIC ATG CTC CAT TGC CA	Hs31869 siglec-1 or sialoadhesin
TAG004	III IIC ATG ACC TCC ACT GG	EST Unigene no. Hs1124557

Results of Confirmation of tag sequences:

Of 18 tag sequences that were analysed with the protocol as described in this example 14 were confirmed with sequences analysis using the RT-PCR with the oligo24dT primer and the tag-based primer as described above (tags designated 004, 007, 011, 012, 014, 015, 016, 017, 022, 025, 029, 030, 032, 036 in table 5). Four tag sequences could not be confirmed based on this method (designated 010, 013, 018, 019). This was probably due to the fact that the tag-based primer was not specific enough or that the polyA tail of the mRNA was not long enough. For one tag an alternative more specific 5'primer was designed to perform a RT-PCR together with -21M13POLYT (tag designated 004). For tag010 a complete specific primer set was designed to perform as well the Reverse Transcription as the amplification. Other tags were confirmed by using a specific RT-PCR primer set followed by a Nested PCR with a specific nested primer set (tags designated 013, 018, 019). Sequence results of all confirmations are listed below and in the figures. The tag sequence in the mRNA sequence if present is shown in bold fonts.

TAG004 (EST AI217565, genbank number BE466728):
Confirmed with protocol from this example.
CATGACCTCCACTGGAAGAGGGGGCTAGCGTGAGCGCTGATTCTCAACCT
ACCATAACTCTTTCCTGCCTCAGGAACTCCAATAAAACATTTTCCATCCA
(Figure 1)

TAG007 (keratin 14, genbank number XM_008578):
Confirmed with protocol from this example.
CATGGATGTGCACGATGGCAAGGTGGTGTCCACCCACGAGCAGGTCCTT
CGCACCAAGAACTGAGGCTGCCCAGCCCCGCTCAGGCCTAGGAGGCCCC
CCGTGTGGACACAGATCCCACTGGAAGATCCCCTCTCCTGCCCAAGCACT
TCACAGCTGGACCCTGCTTCACCCTCACCCCCTCCTGGCAATCAATACAG
CTTCATTATCTGAGTTGCTAAAAAAAAAAAAAAAAAAAAAAAA
(Figure 2)

TAG010 (ephrin A2, genbank number XM_002088):
The RT-PCR with the 5'primer designed on the catg-site (the original primer shown in table 5) gave no confirmation. Most probably the tag-based primer is not specific enough. Ephrin A2 specific primers were used for confirmation. The tag sequence is not included in the Ephrin specific RT-PCR.
ATCTACCAGCTCATGATGCAGTGCTGGCAGCAGGAGCGTGCCCACCGCCC
CAAGTTCGCTGACATCGTCAGCATCCTGGACAAGCTCATTCGTGCCCCTG
ACTCCCTCAAGACCCTGGCTGACTTTGACCCCCGCGTGTCTATCCGGCTC
CCCAGCACGAGCGGCTCGGAGGGGGTGCCCTTCCGCACGGTGTCCGAGT
GGCTGGAGTCCATCAAGATGCAGCAGTATACGGAGCACTTC
(Figure 3)

TAG011 (dual specificity phosphatase, genbank number XM_003720):
Confirmed with protocol from this example.
CATGCTTGACATACCTACCAGTATTATTCCCGACGACACATATACATATG
AGAATATACCTTATTTATTTTTGTGTAGGTGTCTGCCTTCACAAATGTCAT
TGTCTACTCCTAGAAGAACCAAATACCTCAATTTTTGTTTTTGAGTACTGT
ACTATCCTGTAAATATATCTTAAGCAGGTTTGTTTTCAGCACTGATGGAAA
ATACCAGTGTTGGGTTTTTTTTTAGTTGCCAACAGtTGTATGTTTGCTGAT
TATTTATGACCTGAAATAATATATTTCTTCTTCTAAGAAGACATTTTGTTAC
ATAAGGATGACTTTTTTATACAATGGAATAAATTATGGCATTTCTATTG
(Figure 4)

TAG012 (IL1 receptor, type 1, genbank number XM_002686):
Confirmed with protocol from this example.
CATGCATCACGGATCAATAGACTGTACTTATTTTCCAATAAAATTTTCAA
ACTTTGTACTGTT
(Figure 5)

TAG013 (ephrin B1, genbank numbers XM_002535, BC002524):

The RT-PCR with the 5'primer designed on the catg-site (the original primer shown in table 5) gave no confirmation. Most probably the tag-based primer is not specific enough. Ephrin B1 specific primers were used for confirmation. The tag sequence is included in the 3'primer of the Ephrin B1 specific RT-PCR fragment. The Nested PCR fragment is shown here and is just located upstream of the tag sequence.

AACTTGCCCTGTGCCTGTGTCCCCCATGCTAGGGGCGGAGGGGTCTTTTC
CTTCTTCTTTCCTACCTACCCCTTTTCTCTTGGCCAGGGGCCTCGTATCCT
ACCTTTCCTTGTCCCCTGGGCTGGCTGCACAGAGGATTGCCCCTTCTCTTT
TCAGAGCTGGCCCTCGATGCCAAATTAGCATTTAGTATTTTGCTCAAAGTC
TAAGGGACC

(Figure 6)

TAG014 (AT group D protein, genbank numbers XM_006184, AF230388):

Confirmed with protocol from this example.

CATGTTGCATATCAGGGTGCTCAAGGATTGGAGAGGAGACAAAACCAGG
AGCAGCACAGTGGGGACATCTCCCGTCTCAACAGCCCCAGGCCTATGGGG
GCTCTGGAAGGATGGGCCAGCTTGCAGGGGTTGGGGAGGGAGACATCCA
GCTTGGGCTTTCCCCTTTGGAATAAACCATTGGTCTGTCACAAAAAAAAAA
AAAAAAAAAAAAAAA

(Figure 7)

TAG015 (TIE 1, genbank number XM_002037):

Confirmed with protocol from this example.

CATGCCCTGTTCAGCTACTCCCACTCCCGGCCTGTCATTCAGAAAAAAAT
AAATGTTCTAATAAGCTCCAAAAAAAAAAAAAAAAAAAAAAA

(Figure 8)

TAG016 (small ind. Cytokine A18, genbank numbers XM_008451, Y13710, AF111198):

Confirmed with protocol from this example.

CATGGATCAATCAGTGTGATTAGCTTTCTCAGCAGACATTGTGCCATATG
TATCAAATGACAAATCTTTATTGAATGGTTTTGCTCAGCACCACCTTTTAA
TATATTGGCAGTACTTATTATATAAAAGGTAAACCAGCATTCTCAAAAAAA
AAAAAAAAAAA

(Figure 9)

TAG017 (complement comp. 1Q beta, genbank number XM_010666):

Confirmed with protocol from this example.

CATGGAGGGTGCCAACAGCATCTTTTCCGGGTTCCTGCTCTTTCCAGAT
ATGGAGGCCTGACCTGTGGGCTGCTTCACATCCACCCCGGCTCCCCCTGC
CAGCAACGCTCACTCTACCCCCAACACCACCCCTTGCCCAGCCAATGCAC
ACAGTAGGGCTTGGTGAATGCTGCTGAGTGAATGAGTAAATAAACTCTTC
AAGGCC

(Figure 10)

TAG018 (galectin 7, genbank numbers NM_002307, U06648):

The RT-PCR with the 5'primer designed on the catg-site (the original primer shown in table 5) gave no confirmation. Most probably the tag-based primer is not specific enough. Galectin 7 specific primers were used for confirmation. The 5'primer used in the Galectin 7 specific RT-PCR contains the tag sequence. The tag sequence is included in the 5'primer of the Galectin 7 specific RT-PCR fragment. The Nested PCR fragment is shown here and is just located downstream of the tag sequence.

CGGCTGGACACGTCGGAGGTGGTCTTCAACAGCAAGGAGCAAGGCTCCT
GGGGCCGCGAGGAGCGCGGGCCGGGCGTTCCTTTCCAGCGCGGGCAGCC
CTTCGAGGTGCTCATCATCGCGTCAGACGACGGCTTCAAGGCCGTGGTTG

GGGACGCCCAGTACCACCACTTCCGCC
(Figure 11)

TAG019 (FGFR3, genbank numbers NM_022965, NM_000142):
The RT-PCR with the 5'primer designed on the catg-site (the original primer shown in table 5) gave no confirmation. Most probably the tag-based primer is not specific enough. FGFR3 specific primers were used for confirmation. The tag sequence is not included in the Ephrin specific RT-PCR. The Nested PCR fragment is shown here and is located upstream of the tag sequence.
CACAACCTCGACTACTACAAGAAGACAACCAACGGCCGGCTGCCCGTGAA
GTGGATGGCGCCTGAGGCATTATTTGACCGAGTCTACACTCACCAGAGTG
ACGTCTGGTCCTTTGGGGTCCTGCTCTGGGAGATCTTCACGCTGGGGGGC
TCCCCGTACCCCGGCATCCCTGTGGAGGAGCTCTTCAAGCTGCTGAAGGA
GGGC
(Figure 12)

TAG022 (Psoriasin (S100 A7) , genbank number XM_048120):
Confirmed with protocol from this example.
CATGGAGCAGCGCCCTGTTCCGGGGGCAGCCAGTGACCCAGCCCCACC
AATGGGCCTCCAGAGACCCCAGGAACAATAAAATGTCTTCTCCCACC
(Figure 13)

TAG025 (EST Unigene no. Hs173789, genbank numbers XM_018404, AL137262):
Confirmed with protocol from this example.
CATGTCTGCACTGAGAAACTGCATTTCAGTAGCATTTGTCATCCAGCCG
GAAGTTAAAGCACACTTACTTTATTCACCTATTTTTATAATAAACGTTCTT
GCTGCTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
(Figure 14)

TAG029 (PIG, genbank numbers XM_011453, AJ251830):
Confirmed with protocol from this example.
CATGCCACAGGAGAATTCGGGGATTTGAGTTTCTCTGAATAGCATATAT
ATGATGCATCGGATAGGTCATTATGATTTTTTACCATTTCGACTTACATAA
TGAAAACCAATTCATTTTAAATATCAGATTATTATTTTGTAAGTTGTGGAA
AAAGCTAATTGTAGTTTTCATTATGAAGTTTTCCCAATAAACCAGGTATTC
TAAACTTGAAAAAAAAAAAAAAAAAAAAAAAA
(Figure 15)

TAG030 (EST Unigene no. Hs46987, genbank numbers DG151190, BG057289, BE858276, AV681759, BE503169):
Confirmed with protocol from this example.
CATGCTGTGCGGAACTGCGTCAGGGCAAATGTCACAGCAGGATTTCCCC
AACCCAGCTCCATCATCACAGACACAGAGGGCTGCAGGGGAGGCCTGCCC
ACTGTTTTGTCGACTCTGCCCTCCTCTGGCAGCATAGATCCTTAGGTGCTC
AATAAAGGTGTGCTGTATTGAAAAAAAAAAAAAAAAAAAAAAAA
(Figure 16)

TAG032 (SialoAdhesin, also called Siglec 1, genbank number XM_016245):
Confirmed with protocol from this example.
CATGCTCCATTGCCAGACTCTTGCTGGGAGCCCGTCCAGAATGTCCTCC
CAATAAAACTCCATCCTATGACGCAAAAAAAAAAAAAAAAAAAAAAAA
(Figure 17)

TAG036 (Desmoplakin, genbank numbers XM_004463, NM_004415, AF139065):
Confirmed with protocol from this example.
CATGACAGCGGCAATCTTTTCTTTGGTCAAAGTTTTCTGTTTATTTTGCT
TGTCATATTCGATGTACTTTAAGGTGTCTTTATGAAGTTTGCTATTCTGGC
AATAAACTTTTAGACTTTAAAAAAAAAAAAAAAAAAAAAAAA

(Figure 18)

Example 10

Determination of the gene expression levels of the tag sequences in skin samples

To get a feeling for the use of the tag sequences as markers for angiogenesis process skin samples with (5 different samples) and without (2 control samples) Kaposi's Sarcoma lesions were analysed for the expression level of the genes identified by the tag sequences.

Procedure:

1 ml TRIzol reagent (Invitrogen Life Technologies, cat. no. 15596) is added to 10-100 mg tissue or approximately 10^7 cells immediately (tissue is sliced 14μm thick by microtome).

The Total RNA isolation of the samples is performed according to the manufacturers protocol as follows:

- Add 0.2 ml of Chloroform (Merck) and shake the tube vigorously by hand for 15 seconds.
- Incubate for 5 minutes at RT.
- Centrifuge the sample at no more than 12,000 x g for 15 minutes at 4°C.
- Transfer 600μl of the colourless upper aqueous layer to a new tube. The lower organic layer should be red.
- Ad 0.5 ml isopropyl alcohol (Merck) and mix.
- Incubate at room temperature for 10 minutes.
- Centrifuge at no more than 12,000 x g for 15 minutes at 4°C.
- Discard the supernatant and wash the RNA pellet with 1 ml 80% ethanol by vortexing and centrifuge at no more than 7,500 x g at 4°C for 5 minutes and discard the supernatant.
- Place the tube at 56°C for 3 minutes to dry the pellet and proceed with the DNase treatment as described in the next section.

To make sure no genomic DNA exists in the Total RNA isolate we perform a DNase treatment. Protection of RNA against RNase activity of DNase I is done by the addition of RNasin to the DNase reaction. The DNase treatment was performed as follows:

- After TRizol Total RNA isolation resuspend the pellet in 88 µl dH_2O.
- Add sequentially to the RNA solution:
  - 10 µl 10x DNase Buffer (Ambion)
  - 1 µl 40U/µl RNasin (Roche)
  - 1 µl 10 U/µl DNase I (Roche)
- Incubate at 37°C for 1 hr.
- Raise sample volume to 200µl by adding 100 µl dH_2O.
- Add 200 µl cold Phenol/Chloroform pH8 (PC8) and mix thoroughly.
- Centrifuge full speed for 5 minutes at room temperature in a microcentrifuge.
- Transfer the aqueous top layer to a new microcentrifuge tube add sequentially:
  - 3 µl glycogen (Roche)
  - 100 µl 10 M ammonium acetate
  - 700 µl 100% ethanol
- Mix thoroughly and centrifuge at 25,000 x g for 15 minutes at 4°C and decant the supernatant.
- Wash twice by adding 700 µl 80 % ethanol. Mix thoroughly and centrifuge at 25,000 x g for 5 minutes at 4°C and decant the supernatant.
- Dry the pellet for 5 minutes at 56°C and resuspend in 11 µl dH_2O.
- Dilute 1 µl of the RNA isolate in 140 µl dH_2O and calculate the RNA concentration and yield of the isolate through OD_{260} measurement. The yield of DNase treated Total RNA should at least be 13.5µg for the determination of the complete TAG expression profile.

- Prepare a solution of the RNA isolate with a concentration of 50 ng/µl. This is the starting RNA solution for the series of dilution for the TAG specific RT-PCR.

Per sample of DNase treated Total RNA 18 TAG specific RT-PCR/Nested PCR reactions are performed in series of dilution of the RNA. For each sample five control RT-PCR's will be performed in series of dilution namely HIV-1 GAG (SK39/145), GAPDH +RT (in duplo) and no-RT reaction (in duplo). The following dilution scheme was used to derived the samples in serial dilution that are analysed with the RT-PCR:

1. 270 µl 50 ng/µl DNase treated Total RNA DNase treated.
   10 µl 50 ng/µl solution as input in RT-PCR of each primerset = 500ng.
2. 27 µl 50ng/µl Total RNA 10x diluted = 270 µl 5 ng/µl.
   10 µl 5 ng/µl solution as input in RT-PCR of each primerset = 50 ng.
3. 27 µl 5 ng/µl Total RNA 10x diluted = 270 µl 0.5 ng/µl.
   10 µl 0.5 ng/µl solution as input in RT-PCR of each primerset = 5 ng.
4. 27 µl 0.5 ng/µl Total RNA 10x diluted = 270 µl 0.05 ng/µl.
   10 µl 0.05 ng/µl solution as input in RT-PCR of each primerset = 0.5 ng.
5. 27 µl 0.05 ng/µl Total RNA 10x diluted = 270 µl 0.005 ng/µl.
   10 µl 0.005 ng/µl solution as input in RT-PCR of each primerset = 0.05 ng.
6. 27 µl 0.005 ng/µl Total RNA 10x diluted = 270 µl 0.0005 ng/µl.
   10 µl 0.0005 ng/µl solution as input in RT-PCR of each primerset = 0.005 ng.
7. 27µl 0.0005 ng/µl Total RNA 10x diluted = 270 µl 0.00005 ng/µl.
   10 µl 0.00005 ng/µl solution as input in RT-PCR of each primerset = 0.0005 ng.
8. 27 µl 0.00005 ng/µl Total RNA 10x diluted = 270 µl 0.000005 ng/µl.

10 µl 0.000005 ng/µl solution as input in RT-PCR of each primerset = 0.00005 ng.

TAG specific RT-PCR on Total RNA series of dilution using AMV-RT was performed as follows. The reverse Transcription Reactions of all the TAGs on DNase treated Total RNA in series of dilution are performed in 96 wells PCR plates. Ten µl of each Total RNA dilution is used as input for the RT PCR. The reaction volume of the Reverse Transcription is 20 µl and contains dNTP's, MgCl2 and RNasin.

- Prepare the RT-mix per reaction as follows:

3' primer (100 ng/µl) see table 6	1.25 µl
10 x RT buffer	2.0 µl
100 mM dNTP (Pharmacia)	0.8 µl
20 U RNAsin (Roche)	0.3 µl
dH_2O (Baker)	0.65 µl

- Add 10 µl of Total RNA dilution to 5 µl of RT-mix.
- To anneal the primer to the template incubate the reaction mixture at 65°C for 5 minutes followed by cooling down to room temperature.
- Add 5µl 1U/µl AMV-RT to the reaction mixture and perform the Reverse Transcription by incubating at 42°C for 45 minutes.
- After the Reverse Transcription immediately incubate mixture at 95°C for 5 minutes to stop the reaction. Then let the reaction cool down to room temperature.
- Add 80 µl of PCR-mix to each reaction mixture (total volume is 100 µl). Prepare the PCR-mix per reaction as follows:

5' primer (100 ng/µl) see table 6	1.0 µl
10x PCR buffer	8.0 µl
100 mM $MgCl_2$	1.9 µl

Amplitaq 5U/µl	0.4 µl
dH_2O (Baker)	68.7µl

- PCR amplification is performed in a 9700 DNA thermal cycler (Perkin Elmer) according to the following program.

- 5 minutes 95 °C
- 1 minute 95 °C; 1 minute 55 °C; 2 minutes 72 °C, for 35 cycles
- 10 minutes 72 °C

Subsequent to this first round of amplification a second round, nested, amplification was performed. TAG specific second round nested PCR on RT-PCR product was performed as follows:

Add 5 µl of the TAG RT-PCR product in 45 µl of the Nested-PCR mix.

- Prepare the Nested-PCR mix per reaction as follows:

5' nested primer (100 ng/µl), see table 7	0.5 µl
3' nested primer (100 ng/µl), see table 7	0.5 µl
10x PCR buffer	5.0 µl
100 mM $MgCl_2$	1.25 µl
100 mM dNTP (Pharmacia)	0.4 µl
Amplitaq 5U/µl	0.2 µl
dH_2O (Baker)	37.15µl

The combination of primers used for amplification of the genes identified by the tags and the length of the amplified fragment is given in table 8.

- PCR amplification is performed in a 9700 DNA thermal cycler (Perkin Elmer) according to the following program.

- 5 minutes 95 °C
- 1 minute 95 °C; 1 minute 55 °C; 2 minutes 72 °C, for 25 cycles

- 10 minutes 72 °C

- Run 10 μl of the Nested-PCR product on a 1.5% agarose 1xTBE gel stained with EthidiumBromide.
- Visualization of the TAG amplified fragments in the dilution series on a UV-illuminator reveals the level of expression by determining the highest dilution still giving a positive signal.

Table 6. RT-PCR primer design for first round amplification.

Primer	Sequence
5'TAG004GENE	GGC CTT TAA CAC CCC GTT CCT
3'TAG004GENE	TGG TAG GTT GAG AAT CAG CGC TCA
5'TAG007GENE-N	AGG AGA CCA AAG GTC GCT ACT GCA
3'TAG007GENE	CAG TTC TTG GTG CGA AGG ACC T
5'TAG010GENE	ATC TAC CAG CTC ATG ATG CAG TGC T
3'TAG010GENE	GAA GTG CTC CGT ATA CTG CTG CAT
5'TAG011GENE	AGT GGG TAC ATC AAG TCC ATC TGA
3'TAG011GENE	CAC TGG TAT TTT CCA TCA GTG CT
5'TAG012GENE	TAA AGT TGT CCT GCT TGA GCT GGA
3'TAG012GENE	GGC ACG TGA GCC TCT CTT TGC AGT
5'TAG013GENE	CTC TAC CCC AGA GGA ATT TAC AGA
3'TAG013GENE	GGG CCA GAC CAA ACA CAG ACC TCT
5'TAG014GENE	GGC AAC AAG CAG AAG GCG GTC A
3'TAG014GENE	TGA TCT TGA GCT GCA GCT GCT CCT
5'TAG015GENE	GAA TGT GCT GGT CGG AGA GAA
3'TAG015GENE	TGG GGC AGC TTT TCA TAG AGC T
5'TAG016GENE	TTC TCT GCC TGC CCA GCA TCA TGA
3'TAG016GENE	TCA GGC ATT CAG CTT CAG GTC GCT
5'TAG017GENE	GTC TCT ACT ACT TCA CCT ACC A
3'TAG017GENE	TGT TGG GGG TAG AGT GAG CGT TGC T
5'TAG018GENE	GCA GGT TCC ATG TAA ACC TGC TGT
3'TAG018GENE	CTG CTC AGA AGA TCC TCA CGG AGT
5'TAG019GENE	GTG ACC GAG GAC AAC GTG ATG AAG A
3'TAG019GENE	CAT GAT CAT GTA CAG GTC GTG TGT
5'TAG022GENE	TGA GCA ACA CTC AAG CTG AGA G
3'TAG022GENE	TCT CTG GAG GCC CAT TGG T
5'TAG025GENE	ATG GGG TCA GGA ACA TCT GGC AGA
3'TAG025GENE	TCC GGC TGG ATG ACA AAT GCT ACT
5'TAG029GENE	CTC AGG TTT ATC TGG GCT CTA TCA
3'TAG029GENE	TCA TAA TGA CCT ATC CGA TGC AT
5'TAG030GENE	CTT GCA AAG ATA GGA GAG GCT CCA
3'TAG030GENE	ATT GAG CAC CTA AGG ATC TAT GCT
5'TAG032GENE	TGC GAA TCA GGG ACC AAC AGG AGA
3'TAG032GENE	TTG GGA GGA CAT TCT GGA CGG GCT
5'TAG036GENE	ATT TAG CAG TAG TTC TAT TGG GCA
3'TAG036GENE	ACT GAT TAG CAC TTC AGA CGC ACT

Table 7. Nested-PCR primer design.

Primer	Sequence
5'TAG004GENE-2	CAT CGA CAA ATT GCG ATC T
3'TAG004GENE-2	CGC TAG CCC CCT CTT CCA GT
5'TAG007GENE-2.1	AGG AGA TGA TTG GCA GCG T
3'TAG007GENE-2	GGA GGA GGT CAC ATC TCT GGA T
5'TAG010GENE-2	CCA AGT TCG CTG ACA TCG T
3'TAG010GENE-2	TGC TGG GGA GCC GGA TAG ACA
5'TAG011GENE-2	GAA GAG AAA GGA CTC AGT GT
3'TAG011GENE-2	AGA TAT ATT TAC AGG ATA GT
5'TAG012GENE-2	AAA TCC AAG ACT ATG AGA
3'TAG012GENE-2	CTT AGT GGC TGG TGA CAG T
5'TAG013GENE-2	AAC TTG CCC TGT GCC TGT GT
3'TAG013GENE-2	GGT CCC TTA GAC TTT GAG CA
5'TAG014GENE-2	CTT CTG CGA GCT GCA TCT CA
3'TAG014GENE-2	TGC AGT GAC AGC TCC GTC T
5'TAG015GENE-2	AGA GGA GGT TTA TGT GAA GA
3'TAG015GENE-2	ACT ATC TCC CAA AGA AGG ACT
5'TAG016GENE-2	TGT CCT CGT CTG CAC CAT
3'TAG016GENE-2	ATG TAT TTC TGG ACC CAC T
5'TAG017GENE-2	GTC ACC TTC TGT GAC TAT GCC T
3'TAG017GENE-2	ACA GGT CAG GCC TCC ATA TCT
5'TAG018GENE-2	CGG CTG GAC ACG TCG GA
3'TAG018GENE-2	GGC GGA AGT GGT GGT ACT
5'TAG019GENE-2	CAC AAC CTC GAC TAC TAC A
3'TAG019GENE-2	GCC CTC CTT CAG CAG CTT
5'TAG022GENE-2	TTC ACA AAT ACA CCA GAC GTG AT
3'TAG022GENE-2	GGG CGC TGC TCC ATG GCT CTG CT
5'TAG025GENE-2	TGC CTA GAA AGG GGT GGC T
3'TAG025GENE-2	TTC TCA GTG CAG ACA TGT GGC T
5'TAG029GENE-2	CAG GCT TCT GAT AGT TTG CAA CT
3'TAG029GENE-2	TAT GCT ATT CAG AGA AAC T
5'TAG030GENE-2	TCT AAT GCA TGT AGA AGC T
3'TAG030GENE-2	AGG GCA GAG TCG ACA AAA CAG T
5'TAG032GENE-2	TCT TGA GTG GGC TAG TGA CT
3'TAG032GENE-2	AGT CTG GCA ATG GAG CAT GA
5'TAG036GENE-2	TGC TAT ACC TTG ACT TCA T
3'TAG036GENE-2	TCC AAG TGT ACT GCT TAT
5'TAG036GENE-2.1	CTA GTA GTC AGT TGG GAG T
3'TAG036GENE-2.1	AGC CAG AAC AGC CTT TAC T

Table 8 Primer combinations and amplified fragment length in the Tag specific Nested PCR reactions

Name	5' primer name	3' primer name	PCR fragment
TAG004	5' TAG004gene-2	3' TAG004gene-2	182
TAG007	5' TAG007gene-2.1	3' TAG007gene-2	211
TAG010	5' TAG010gene-2	3' TAG010gene-2	108
TAG011	5' TAG011gene-2	3' TAG011gene-2	239
TAG012	5' TAG012gene-2	3' TAG012gene-2	197
TAG013	5' TAG013gene-2	3' TAG013gene-2	212
TAG014	5' TAG014gene-2	3' TAG014gene-2	243
TAG015	5' TAG015gene-2	3' TAG015gene-2	131
TAG016	5' TAG016gene-2	3' TAG016gene-2	219
TAG017	5' TAG017gene-2	3' TAG017gene-2	185
TAG018	5' TAG018gene-2	3' TAG018gene-2	175
TAG019	5' TAG019gene-2	3' TAG019gene-2	204
TAG022	5' TAG022gene-2	3' TAG022gene-2	238
TAG025	5' TAG025gene-2	3' TAG025gene-2	183
TAG029	5' TAG029gene-2	3' TAG029gene-2	141
TAG030	5' TAG030gene-2	3' TAG030gene-2	179
TAG032	5' TAG032gene-2	3' TAG032gene-2	223
TAG036	5' TAG036gene-2	3' TAG036gene-2	191

Results

The results of determination of the expression levels of the genes identified by the tag sequences are depicted in figure 19. The data clearly indicate that a number of the genes identified by the tag sequences have a higher expression in the skin samples with Kaposi's Sarcoma lesions compared to normal skin: tag007, tag010, tag012, tag013, tag014,, tag015, tag016, tag017, tag022, tag029, tag030, tag032 and tag036.

Example 11

Determination of the gene expression levels of the tag sequences in peripheral blood mononuclear cell (PBMC) samples

To get a feeling for the use of the tag sequences as markers for angiogenesis process in samples not from the location of the angiogenesis process, i.e. the Kaposi's Sarcoma in the skin or another tumour in the body but at an accessible sample from the blood (PBMC's) the expression of 5 tag identified genes was determined in PBMC samples. The PBMC samples were from the blood of patients with (4 different samples) and without (2 control samples) Kaposi's Sarcoma lesions and were analysed for the expression level of 5 tag identified genes in PBMC's.

The procedure of the example was identical as described in example 10, with the exception that PBMC samples were used (approximately 10 million cells per sample, ranging from 2.5 to 50 million) instead of skin biopsies. The genes that were analysed are identified by tag007, tag017, tag010, tag013, tag015, tag029 and tag032. The results of the analysis are depicted in figure 20. It is clear from these data that the elevated expression in the tumour sites of the genes identified by tag015 (TIE 1) and tag032 (Salioadhesin or Siglec 1) is paralleled in the blood cell fraction, i.e. PBMC.

Results

The data clearly show that the over expression of the gene identified by tag007 (Keratin 14) in skin samples (see example 5) is not paralleled in blood. In contrast, in the samples tested in this example the gene identified by tag007 is not expressed at all in the blood compartment. This shows that no tumour cells expressing tag007 are present in blood. As a consequence, measurement of tag007 in the blood could be a good indicator for the presence of these tumour

cells in the blood, and thus a marker for circulating cancer cells that can cause metastasis.

The up regulation of genes identified by tag015 (TIE 1) and tag032 (Saliodhesin or Siglec 1) in skin samples is clearly paralleled in the blood. These two tags are higher expressed in blood from patients with tumours compared to healthy individuals. This up-regulation is due to up-regulation of expression in typical blood cells. The up-regulation cannot be due to the presence of tumour cells that express tag015 and tag032 in the blood, because the absence of expression of the gene identified by tag007 in the blood shows that no tumour cells are present in blood, as explained above. This means that measurement of expression of tag015 and/or tag032 in the blood indicates the presence of a tumour somewhere else in the body. Furthermore, measurement of expression of genes identified by tag015 and/or tag032 during anti-tumour therapy and/or anti-angiogenesis therapy can be used to monitor the efficacy of this treatment.

<u>Conclusions</u>

The paralleled up-regulation of the genes identified with tag015 (TIE 1) and tag032 (Salioadhesin or Siglec 1) in both the tumour and the blood, enables the monitoring of the efficacy of a therapy aimed at decreasing the growth of a tumour, in particular anti-angiogenic tumour treatment. This follows the reasoning that if these two genes are markers in PBMC for blood vessel formation in a tumour in another site in the body, these two markers in blood will also decrease with the decrease of this blood vessel growth at the tumour site.

The genes identified by tag007 (Keratin 14), tag015 (TIE 1) and tag032 (Salioadhesin or Siglec 1) have different expression in the blood of patients with a tumour compared to normal individuals. Therefore these genes, in particular the expression thereof, can be used to screen a population at risk for the presence of tumour in individual members of that population.

All the genes identified by tags in this study that have changed expression levels comparing normal to tumour tissue, i.e. tag007, tag010, tag012, tag013, tag014, tag015, tag016, tag017, tag022, tag029, tag030, tag032 and tag036 are encoding potential target molecules for therapeutic compound with anti-angiogenic effects applicable in tumour treatment, and/or these genes encode potential target molecules that can be potential target molecules for therapeutic compounds that stimulate the growth of blood vessel, for instance in the treatment of heart and coronary disease.

Brief description of the drawings

Figure 1: Sequence involved in angiogenesis. A change of expression of this sequence after a certain treatment indicates that said treatment is effective. This sequence is identical to an EST sequence identified from human foetal heart (GenBank acc. # AI217565 and others), which in turn matches a predicted exon on chromosome 19. A relation with angiogenesis has not been described previously.

Figure 2-18: Sequences which are identified by name and Genbank numbers (NCBI database). Other identification can be found in tables 1-4 (Unigene numbers) that can be found in the SAGE databases of NCBI.

Figure 19. Mean expression levels and standard deviation depicted as log dilution factor that still is positive starting with 500 ng of total RNA isolated from 5 skin samples with Kaposi's Sarcoma (light bars) and 2 control, normal skin samples (dark bars).

Figure 20. Mean expression levels and standard deviation depicted as log dilution factor that still is positive starting with 500 ng of total RNA isolated from 4 PBMC samples of patients with Kaposi's Sarcoma (light bars) and 2 control, normal PBMC samples (dark bars).